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Short Communication

Structure of fungal polysaccharides isolated from the cell-wall of three strains of *Verticillium fungicola*

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Abstract

The structure of fungal polysaccharides, isolated from the cell-wall of three isolates of *Verticillium fungicola*, has been investigated by chemical analysis and 1D and 2D ^{1}H- and ^{13}C-NMR spectroscopy. The polysaccharides have an idealized repeating unit of:

$$\{\rightarrow 6)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow [6)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow]_{n}\}_{m}$$
$$\begin{array}{c} 4 \\ \uparrow \\ \beta\text{-D-Gal}p \end{array}$$

where *n* ranges from 10 to 15 and *m* from 200 to 240.

Keywords: Fungi; *Verticillium*; Polysaccharides; NMR spectroscopy

1. Introduction

Verticillium fungicola (Preuss) Hassebr. var. *fungicola* is a mitosporic fungi which is one of the most common pathogens of the cultivated mushroom *Agaricus bisporus*, causing the 'dry bubble' disease. This disease was first reported by Constantin and Dufour (1982), and is an unresolved problem in the cultivation of the mushroom. The pathological effects seem to be due to the production of extracellular enzymes (Calonje, García-Mendoza, Galán, & Novaes-Ledieu, 1997; Gray & Morgan-Jones, 1981; Kalberer, 1984; Thapa & Jandaik, 1989), which causes the degradation of the host cell-wall and penetration of the mycoparasite in the host hyphae, followed by degradation of cytoplasm and death of the cell (Calonje et al., 1997; Drag, Gells, De Bruijn, & Van Griesven, 1996)

Connection of the parasite to the host cell surface probably involves binding to the surface polysaccharides, thus, a first step in the understanding of the biochemical events occurring during infection should be the determination of the structure of these polysaccharides.

We have found that the alkali-extractable and water-soluble fungal polysaccharides, which are minor components of the cell-wall (2–8%) and constitute the glucidic moiety of peptido-polysaccharides, differ in composition and structure among genera and, in certain cases, among groups of species of a genus (Leal, Prieto, Ahrazem, Pereyra, & Bernabé, 2001). Due to their diversity, they have also been proposed as chemotaxonomic markers at the genus or subgenus level (Ahrazem et al., 1997; Leal et al., 1997; Prieto et al., 1997) as well as a mean for establishing relationships among teleomorphic genera (perfect state) and their anamorphs (imperfect state). These polysaccharides are antigenically relevant (De Ruiter et al., 1991; Domenech et al., 1996; Latgé et al., 1991) and are probably involved in cell–cell and/or cell–host recognition mechanisms.

The chemical components of the *V. fungicola* cell-wall have been recently investigated (Calonje, Novaes-Ledieu, Bernardo, Ahrazem, & García-Mendoza, 2000), and we report here on the structural study of an alkali-extractable and water-soluble polysaccharide isolated from three strains of *V. fungicola* var. *fungicola*, as an initial attempt to understand the biochemical basis of the host–parasite interaction of the dry bubble disease.

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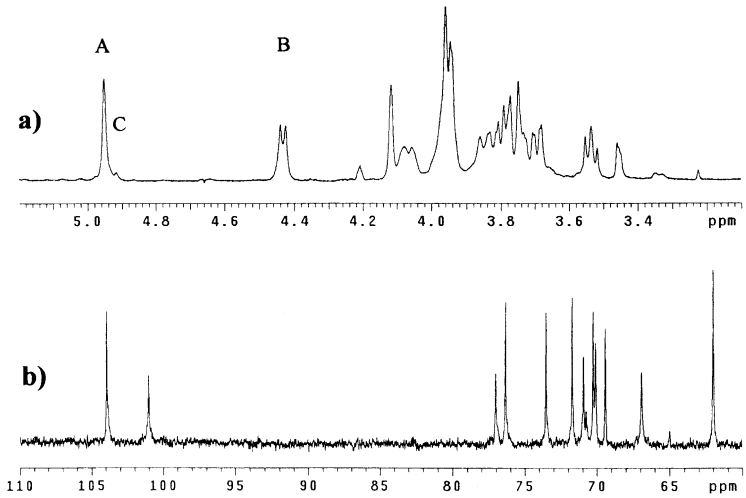


Fig. 1. ^{1}H- and ^{13}C-NMR spectra (500 MHz, D_2O, 35 °C) of the fraction F1SS of the cell-wall polysaccharide isolated from *V. fungicola*: (a) ^{1}H-NMR spectrum, showing the three distinct anomeric protons, which have been labelled **A**–**C**; (b) ^{13}C-NMR spectrum at 125 MHz.

2. Experimental

Microorganisms and culture media: The strains of *V. fungicola* var. *fungicola* used were CBS 992.69, CBS 440.34, and *V. fungicola* CIES (Centro de Investigación, Experimentación y Servicios del Champiñón, Cuenca, Spain), isolated from infected carpophores of *Agaricus bisporus*, obtained from Dr C. García-Mendoza. The microorganisms were maintained on slants of Bacto potato dextrose agar supplemented with 1 g l^{-1} of Bacto yeast extract (Difco). The basal medium and growth conditions for mycelium production have been previously described (Gómez-Miranda, Moya, & Leal, 1988).

Wall material preparation and fractionation: The preparation of wall material (Gómez-Miranda, Prieto, & Leal, 1990) and the fractionation procedure (Leal, Guerrero, Gómez-Miranda, Prieto, & Bernabé, 1992) were performed as previously described. The crude polysaccharidic preparation extracted from the dry cell-wall material with 1 M NaOH at 20 °C contained water-soluble polysaccharides (fraction F1S) and water insoluble polysaccharides (F1I). Fractions F1S were refractionated by treatment with a small portion of water (about 50 mg ml^{-1}), followed by centrifugation (10,000 × *g*, 30 min), giving a solution (F1SS), and a precipitate (F1SI). F1I and F1SI were not further investigated.

For preparative chromatography, 100 mg of fraction F1SS were processed according to Ahrazem et al. (1997).

Chemical analyses: Neutral sugars were released by treating the samples overnight with methanolic 0.6 M HCl at 80 °C and hydrolysing with 3 M trifluoroacetic acid for 1 h at 121 °C. The hydrolysis products were reduced with sodium borohydride and the corresponding alditols were acetylated with pyridine–acetic anhydride (1:1) for 1 h at 100 °C (Laine, Esselman & Sweeley, 1972). Identification and quantification were carried out by gas–liquid chromatography (GLC) using a fused silica SP-2380 column (30 m × 0.25 mm, 0.2 μm film thickness) and a temperature program (210–240 °C, 3 min initial hold, 15 °C min^{-1} ramp rate and 7 min final time). The absolute configuration of the sugars was determined as devised by Gerwig, Kamerling, and Vliegenthart (1978).

Partial hydrolysis: The polysaccharide F1SS was treated with 0.05 M H_2SO_4 at 100 °C for 8 h, and the product dialysed against water (M_w cutoff = 1000 Da). The retentate was recovered and lyophilised for further analysis.

Methylation analysis: The polysaccharide was methylated following the method by Ciucanu and Kerek (1984) and hydrolysed sequentially with 90% formic acid in water at 80 °C for 2 h and 5 M TFA at 100 °C for 5 h. The products were reduced with $NaBD_4$ and then acetylated as earlier. The corresponding partially methylated alditol acetates were examined by GC–MS using a fused silica SPB-1 column (30 m × 0.25 mm, 0.2 μm film thickness), a temperature program (160–210 °C, 1 min initial hold, 2 °C min^{-1} ramp rate) and a mass detector Q-mass from Perkin–Elmer. Quantification was made according to peak area.

NMR analysis: Polysaccharides F1SS (~20 mg) were dissolved in D_2O (0.8 ml) followed by centrifugation (10,000 × *g*, 20 min). The supernatants (ca. 0.7 ml) were used for recording ^{1}H-NMR spectra. The column-purified sample for 2D experiments was lyophilised, redissolved in D_2O (1 ml) and the process repeated twice for further deuterium-exchange. The final sample was dissolved in 0.7 ml of D_2O (99.98% D). 1D- and 2D-^{1}H- and ^{13}C-NMR experiments were carried out at 35 °C on a Varian Unity 500 spectrometer with a reverse probe and a gradient unit. Proton chemical shifts refer to residual HDO at δ 4.66 and carbon chemical shifts to internal acetone at δ 31.07.

The 2D-NMR experiments (DQF-COSY, 2D-TOCSY, HMQC, and HMBC) were performed by using the standard Varian software, as described previously (Jiménez-Barbero, Bernabé, Leal, Prieto, & Gómez-Miranda, 1993).

Table 1
^{1}H- and ^{13}C-NMR chemical shifts (δ) and proton coupling constants for the alkali-extractable and water-soluble cell-wall polysaccharide isolated from *Verticillium fungicola* (underlined bold numbers represent glycosylation sites)

Residue		1	2	3	4	5	6a	6b
A	H	4.95	4.12	3.97	ca. 3.94	3.96	4.04	3.86
	C	101.0	70.1	70.3	**77.0**	70.9	**66.9**	–
B	H	4.43	3.54	3.69	ca. 3.96	3.74	ca. 3.80	ca. 3.76
	C	104.0	71.7	73.5	69.4	76.4	62.0	
C	H	4.92	4.06				4.11	3.93
	C	100.3	70.9				**67.0**	–

3. Results and discussion

Fractions F1SS from the three strains (see Section 2) consisted of mannose and galactose, as shown by GLC of their alditol acetates. Their ^{1}H-NMR spectra in D_2O were compared, showing that the structure of all of them were practically identical. The polysaccharide from *V. fungicola* var. *fungicola* CIES was then purified by column chromatography for further studies.

Methylation analysis gave two main peaks identified as 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-galactitol, which corresponds to terminal galactopyranose, and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-mannitol, which can derive from either a 4,6-di-*O*-substituted mannopyranose or a 5,6-di-*O*-substituted mannofuranose. In addition, a small quantity of 6-*O*-substituted Man*p* was detected.

The high resolution ^{1}H-NMR spectrum of the polysaccharide contained two main signals in the anomeric region at 4.95 ppm ($J_{1,2} < 1.5$ Hz) and 4.43 ppm ($J_{1,2} = 7.4$ Hz), which were assigned to the mannose and galactose residues, respectively, and a small signal at 4.94 ppm. The corresponding residues were labelled **A**, **B**, and **C**, according to their anomeric peaks (see Fig. 1a). The ^{13}C-NMR spectrum contained two anomeric peaks (104.0 and 101.0 ppm) (Fig. 1b). Neither the value of the proton anomeric coupling constant of the mannose residue ($J_{1,2} < 1.5$ Hz) nor that of its anomeric carbon chemical shift were in accordance with those expected for a furanose ring ($J_{H\text{-}1,H\text{-}2}$ = ca. 3.0 Hz for α and ca. 4.9 Hz for β anomers; $\delta_{C\text{-}1\alpha}$ = ca. 103 ppm; $\delta_{C\text{-}1\beta}$ = ca. 109 ppm) (Bock & Pedersen, 1983; Cyr & Perlin, 1979).

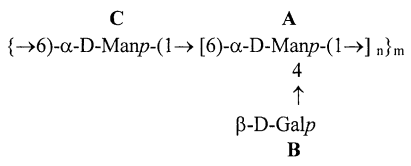
In addition, a partial hydrolysis of the polysaccharide with 0.05 M H_2SO_4 at 100 °C for 8 h led to elimination of a considerable amount of the Gal*p* residues, giving a new polysaccharide which consisted of over 85% of 6-*O*-substituted Man*p* and a small quantity of 4,6-di-*O*-substituted Man*p* and terminal Gal*p*.

The ^{1}H- and ^{13}C-NMR spectra were assigned using ^{1}H–^{1}H homonuclear correlation experiments (DQCOSY, TOCSY) and proton-detected ^{1}H–^{13}C heteronuclear correlation experiments (HMQC, HMBC, and HSQC–TOCSY). The data obtained for residues **A** and **B** are shown in Table 1. The values observed for C-4 and C-6 of residue **A** are shifted downfield with respect to those reported for unsubstituted Man*p* units (Bock & Pedersen, 1983), thus confirming the analytical findings. With respect to unit **C**, the information obtained from NMR experiments was very poor, due to severe overlapping of signals, both for proton and carbon spectra. However, from the methylation results, it seems obvious that **C** is 6-*O*-substituted-Man*p*.

Concerning the configuration of the mannose units, a coupled HMQC experiment allowed the measurement of the values for $^{1}J_{C\text{-}1,H\text{-}1}$ in units **A** and **C** (176 Hz for both) which, in accordance with the values of chemical shifts, indicated α-Man*p* configuration for both of them. In unit **B**, the value of $^{1}J_{C\text{-}1,H\text{-}1} = 160.3$ Hz, was in agreement with a β-configuration for the Gal*p*, already deduced from the anomeric coupling constant of the proton spectrum.

To discriminate between the two possibilities of arrangement of the glycosylation sequence (namely, a backbone of α-(1 → 6)-Man*p*, substituted at position 4 of each residue by terminal β-Gal*p*, or (less likely) a backbone of α-(1 → 4)-Man*p*, substituted at positions 6 by the Gal*p* moiety, with a few α-(1 → 6)-Man*p* linkages) we performed a long-range proton–carbon correlation HMBC experiment, which showed signals for H-1 **A**/C-6 **A**′(**C**), H-1 **B**/C-4 **A**, and H-4 **A**/C-1 **B**, H-6 **A**′/C-1 **A**(**C**), (where **A**′ means a second unit of 4,6-di-*O*-α-substituted-Man*p*) demonstrating the occurrence of the first sequence.

The results from methylation analysis, together with the NMR spectral data, suggest the following idealised structures for the F1SS cell-wall polysaccharide of those isolates:



being *n* around 15 in *V. fungicola* var. *fungicola* CIES, and around 10–12 in the other two strains. A small amount of three different methoxyl groups were detected ca. 3.46 ppm (C = 55 ppm). The low proportion of these methoxyl groups (around one for every 30 pyranose units) greatly difficulted their exact localisation, and they were not further investigated. The average molecular mass of the polydisperse polysaccharide is in the range of 70–80 kDa, as calculated by gel permeation chromatography on a Sepharose CL-6B column, previously calibrated with different dextrans. Then, the value of *m* is around 200–240.

Our results add new support to the proposition that the alkali-extractable water-soluble fungal polysaccharides, in addition to their immunological relevance, may be useful as chemotaxonomic characters (Leal, Prieto, Ahrazem, Pereyra, & Bernabe, 2001) and as a source of new polysaccharide structures.

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